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13. ABSTRACT ***(Maximum 200 Words)***

We have preliminary evidence that both PI3K and PAK are differentially activated in the highly metastatic Met-1 tumors compared with the low metastatic Db-7 tumors. Furthermore, using micro array analysis, osteopontin (OPN), a protein linked to metastatic breast cancer in humans is differentially up regulated in Met-1 tumors compared with Db-7 tumors. We have confirmed the over expression of this gene product in Met-1 tumors by immunohistochemistry and immunoblot analysis. Further analyses are necessary to establish whether there is a linkage between PI3K/PAK activation and OPN expression. Preliminary data suggests that PyMT is capable of modulating the endocytosis of proteins expressed at the cell surface. Further study is required to confirm this observation.

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Table of Contents

5. Introduction

Metastatic progression of tumors is a complex, multifactorial process that involves various steps such as cellular migration, implantation, neovascularization, and tissue colonization. The molecular sequence of events that are necessary metastatic conversion is poorly understood. To better understand the mechanism(s) of breast cancer metastasis, we are studying the signaling pathways that control metastatic development. The focus of this project is to determine whether phosphatidylinositol 3-kinase (PI3K) or related signaling pathways are critical for metastatic progression. We hypothesize that PI3K links uncontrolled growth to the cell processes necessary for metastasis. We are utilizing a unique model system to study breast cancer metastasis. Specifically, we are examining two different genetically altered breast cancer tumor lines [called Met-1/Db-7 that are transformed with polyomavirus middle T-antigen (PyMT)] that vary in their metastatic potential when implanted into mice. Met-1 tumors are highly metastatic and are transformed by wild-type PyMT. In contrast, Db-7 tumors exhibit a low metastatic frequency and are transformed by a PyMT mutant tha abrogates the ability of PyMT to bind and activate PI3K.
Consequently, we are examining the signaling pathways that emanate from PyMT with a particular focus on the PI3K pathway, to determine whether these intracellular signaling pathways are critical for metastatic development. Furthermore, we are testing our hypothesis that PI3K activation is a critical determinant for metastatic development by transducing dominant-negative and constitutively active forms of PI3K and other signaling factors that are differentially activated or expressed in the Met-1 and Db-7 tumor lines. These transduced tumor lines will be assessed for their ability to metastasisze after implantation of the tumors into the mammary fat pads of female nude mice. Thus, this projects addresses the *in vitro* characterization of the PyMT transformed cells and the *in vivo* characterization of tumors transduced with genetic mutants of signaling proteins.

6. Body

Statement of Work

The Specific Aims of the proposal are as follows:
(1) Determine whether the difference in metastatic potential of the metastatic (Met-1) and non-metastatic (Db-7) mouse mammary tumor lines are due to alterations in PI3K activation and/or the signalling intermediates that emanate from the PI3K activation pathway.
(2) Determine whether disruption of the PI3K activation pathway affects the metastatic and non-metastatic phenotypes *in vitro*. To achieve this goal, dominant-negative and constitutively-active mutants of PI3K and/or signalling intermediates in the PI3K pathway will be inducibly expressed using a novel tetracycline-inducible retroviral vector.
(3) Analyze the metastatic potential of the tumor lines that inducibly express the transduced dominant-negative and constitutively-active regulatory mutants of PI3K and/or its signalling intermediates *in vivo.*

Activation of PI3K signaling in Polyoma middle t-antigen transformed cells

Using *PI3K* assays, we have found that PI3K is activated by polyoma middle T-antigen transfected into murine Balb/c cells. This activation of PI3K was inhibited by treatment with 0.1mM wortmannin a PI3K inhibitor (Fig. 1).

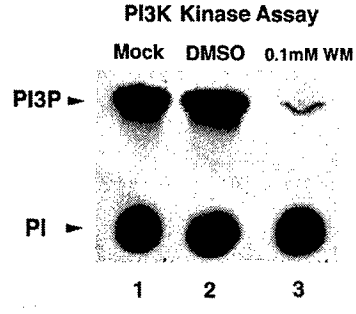


Fig. 1. To prove that PI3K was activated in murine cells transformed by polyomavirus middle T-antigen, PI3K assay was performed on PI3K immunoprecipitates. PI3K immunoprecipitates were either untreated (lane 1, mock), or treated with DMSO (lane2) or 0.1mM wortmannin (WM).

Further analyses were performed using the highly metastatic Met-1 and low metastatic Db-7 cells to determine whether PI3K was activated in these tumors. The results from these analyses demonstrated that PI3K was indeed activated in the Met-1 lines compared to the Db-7 lines. These results are consistent with previous studies that show that the double base mutation of polyomavirus middle T-antigen abrogates the interaction between PyMT and PI3K.

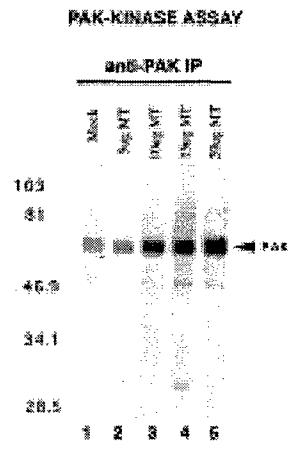


Fig. 2

Polyoma middle T-antigen activates p21-Activated Kinase

It is likely that downstream signaling events important for metastatic progression. We tested whether PyMT was capable of activating PAK activation by performing in vitro kinase assays on PAK immunoprecipitates from murine cells transfected with PYMT (Fig. 2). We found that PAK was very strongly activated by PyMT. P21-activated kinases (PAK, a serine-threonine kinase) are are activated through interactions with Ras-like G-proteins (Rac and Cdc42). These kinases also interact with guanine nucleotide exchange factors (Pix and Cool) and adapter proteins (Nck). PAK proteins have been shown to mediate growth factor-induced morphological changes involving actin-based cellular structures (i.e., formation of membrane ruffles and peripheral filopodia). These changes are critical for cellular mobility and locomotion. PAKs also modulate gene expression through activation of the Jun kinase (Jnk) signaling pathway. These results indicate that PAK activation is downstream of PyMT. We have preliminary evidence that PAK is differentially activated in the highly metastatic Met-1 tumors compared with the low metastatic Db-7 tumors.

Osteopontin is differentially expressed in Met-1 tumors.

In collaboration with Dr. Jeff Gregg (Department of Medical Pathology, UC Davis), subtractive microarray analysis was performed on Met-1 and Db-7 tumors to determine whether specific genes were differentially expressed in metastatic versus non-metastatic tumors. Microarray analysis revealed that a gene that had previously been identified in metastatic breast cancer, osteopontin (OPN), was differentially expressed in the Met-1 tumors compared with the non-metastatic Db-7 tumors. This finding was supported by quantitative RT-PCR analysis that was performed on the Met-1 and Db-7 tumors (Fig 3).

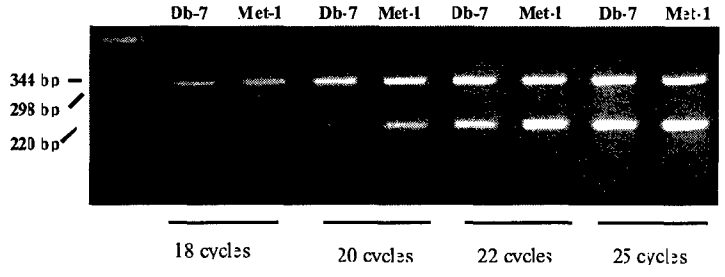


Fig. 3. Quantitative RT-PCR analysis

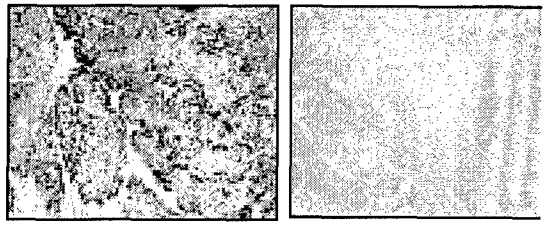


Fig. 4. Immunohistochemical analysis of Met-1/Db-7 tumors using OPN monoclonal antibody

To confirm that OPN was differentially expressed in Met-1 tumors, immunohistochemistry (Fig. 4) and immunoblot analysis (Fig. 5) was performed on the Met-1 and Db-7 tumors. These results confirm that OPN is differentially expressed in the highly metastatic Met-1 tumors compared with the non-metastatic Db-7 tumors.

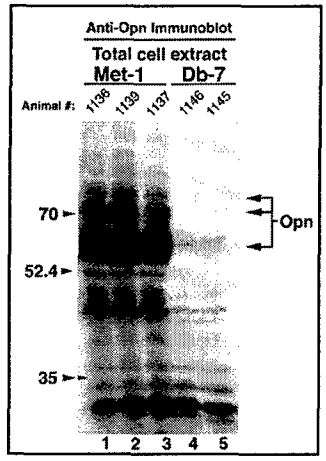
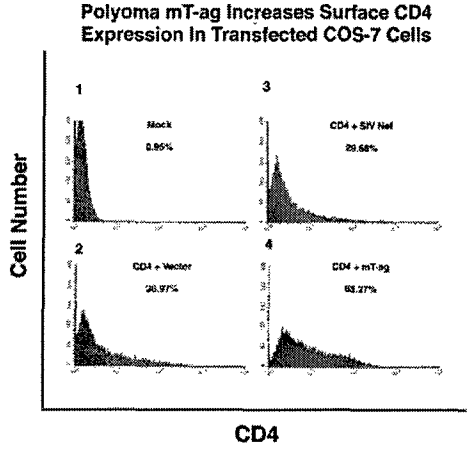


Fig. 5. Total cell extracts were prepared from three Met-1 and two Db-7 tumors from different mice (animal numbers are indicated). Proteins were separated on SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was performed with a monoclonal antibody to OPN and detected by ECL. The position of OPN is indicated on the right.

The results of these experiments reveal that Met-1 preferentially expresses osteopontin. These results are consistent with those from previous studies that indicate OPN is associated with metastatic breast cancer in humans. We are currently investigating whether the differential OPN expression is regulated by PI3K activation.

Suppression of CD4 endocytosis by PyMT



HIV-1 and SIV Nef, a regulatory gene product necessary for AIDS pathogenesis, is known to bind and activate PAK and down regulate the expression of CD4 from the surface of Cos-7 cells cotransfected with CD4 and Nef. In our CD4-down regulation studies using Nef mutants, we included PyMT as a "negative" control. Unexpectedly, we found that the expression of PyMT enhanced the expression of CD4 on the cell surface by greater than 2-fold, as determined by FACS analysis. This is in contrast to Nef which down regulates the expression of CD4 from the cell surface by 30-40%. Because Nef has been shown to accelerate the endocytosis and degradation of CD4 expressed on the cell surface via the clathrin coated pit pathway, it is likely that PyMT is inhibiting the endocytosis of CD4. Further analyses are in-progress to determine whether this inhibition is a specific or generalized effect involving other cell surface markers (such as cell adhesion molecules). We can conclude from our experiments that this effect is probably not due to PyMT effect on PI3K activation, however, additional studies using PyMT mutants that knock-out Src or PI3K association are in progress. These results may have important implications on the trafficking of cell surface molecules. If this property of PyMT affects tumor cell adhesion, this may reveal clues as to why cells transformed by PyMT (i.e., Met-1) may be highly metastatic.

7. Key Research Accomplishments

We have begun to characterize the intracellular signaling pathways that are influenced by the polyomavirus oncogene Middle T-antigen. Both PI3K and PAK are activated by PyMT in murine cells. We have preliminary evidence that both PI3K and PAK are differentially activated in the highly metastatic Met-1 tumors compared with the low metastatic Db-7 tumors. Furthermore, using micro array analysis, OPN, a protein linked to metastatic breast cancer in humans is differentially up regulated in Met-1 tumors compared with Db-7 tumors. We have confirmed the over expression of this gene product in Met-1 tumors by immunohistochemistry and immunoblot analysis. Further analyses are necessary to establish whether there is a linkage between PI3K/PAK activation and OPN

expression. Preliminary data suggests that PyMT is capable of modulating the endocytosis of proteins expressed at the cell surface. Further study is required to confirm this observation.

8. Reportable Outcomes

- Both phosphatidylinositol 3-kinase (PI3K) and p21 activated kinase (PAK) are activated by polyoma Middle T-antigen (PyMT) in murine cells.
- Osteopontin (OPN) is differentially expressed in the highly metastatic Met-1 tumors as compared with the low metastatic Db-7 tumors.
- Polyoma middle T-antigen appears to influence the endocytosis of specific molecules at the cell surface.

9. Conclusions

We have found that the cellular serine-threonine kinase p21-Activated Kinase is activated in Met-1 tumors and in murine cells transformed with the wild-type form of PyMT. We have evidence that PI3K is activated in the highly metastatic Met-1 cells compared with the low metastatic Db-7 cells. We are currently determining whether the activation of PAK by PyMT is dependent on PI3K activation. The activation of PAK by PyMT represents a previously unrecognized signaling pathway affected by PyMT that influences cell shape, morphology, and intracellular trafficking. We have also found that PyMT appears to inhibit the endocytosis of cellular proteins from the cell surface. The effect of PyMT on trafficking of surface receptors has not been described previously. The inhibition of receptor endocytosis may serve to prolong the ability of signaling molecules to maintain a proliferative or activation signal. Further studies regarding the role of PI3K activation on endocytosis and trafficking of surface proteins need to be performed to establish whether a linkage exists. Consistent with the idea that PI3K activation is necessary for metastatic conversion, is the observation that osteopontin (OPN) is differentially expressed in the highly metastatic Met-1 tumors compared with the low metastatic Db-7 tumors. These results provide a basic framework to examine the intracellular signaling pathways that are affected by metastatic development and progression. Results from this project may help in the development of new therapeutic approaches to prevent the progression of metastatic breast tumors as well as identify markers that would help in the detection and diagnosis of metastatic breast cancer.

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11. Appendicies

Abstracts

Hamza, M.S., Cheung, A.T., Cardiff, R.D., and **Sawai, E.T.** Activation of Phosphotidylinositol 3'-kinase and p21-Activated kinase in Murine cells Transformed by Polyoma Middle T-antigen. Fourth Annual Cancer Research Symposium, UC Davis, September, 1999.

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